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RESEARCH PAPER

Preparation, Drug Release, and Cell Growth Inhibition of a Gelatin: Doxorubicin Conjugate

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ABSTRACT

Purpose To demonstrate the feasibility of a novel macromolecular delivery system for doxorubicin (DOX) which combines pH dependent DOX release with a high molecular weight and biodegradable gelatin carrier.

Methods DOX was conjugated to gelatin using an acid labile hydrazone bond and a glycylglycine linker. The gelatin-doxorubicin conjugate (G-DOX) was evaluated for hydrazide and DOX content by spectrophotometry, molecular weight by HPLC-SEC, *in vitro* DOX release at various pH, and cell growth inhibition using EL4 mouse lymphoma and PC3 human prostate cells.

Results G-DOX hydrazide and DOX content was 47% and 5–7%, respectively of theoretical gelatin carboxylic acid sites. During preparation of G-DOX, the molecular weight decreased to 22 kDa. DOX release was 48% in pH 4.8 phosphate buffer, 22% at pH 6.5, but 10% at pH 7.4. The G-DOX IC50 values in EL4 and PC3 cells were 0.26 μM and 0.77 μM, respectively; the latter value 3 times greater than that of free DOX.

Conclusions A 22 kDa macromolecular DOX conjugate containing 3.4–5.0% w/w DOX has been prepared. The pH dependent drug release in combination with a biodegradable gelatin carrier offer potential therapeutic advantages of enhanced tumor cell localization and reduced systemic toxicities of the drug.

KEY WORDS acid labile hydrazone · cell growth inhibition · doxorubicin · drug delivery · gelatin · HPLC-SEC · macromolecular conjugates

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ABBREVIATIONS

DOX	doxorubicin
EDC	1-ethyl-3-(dimethylaminopropyl) carbodiimide HCl
G-DOX	gelatin – doxorubicin conjugate
GGBE	glycylglycine benzyl ester p-toluene sulfonate
IC_{50}	concentration of agent to inhibit growth to 50%

INTRODUCTION

Doxorubicin (DOX) is a potent antineoplastic agent that is effective against a wide range of solid tumors and lymphomas but it is also associated with an irreversible cardiomyopathy above cumulative doses of 550 mg/m^2 (1). This and other toxic side effects make the drug a good candidate for localized drug delivery. DOX has been investigated in several macromolecular delivery systems such as liposomes (2), synthetic copolymers of N-(2-hydroxypropyl)methacrylamide (HPMA) (3,4) other synthetic water soluble polymers (5), micelles (6,7), polysaccharides (8) as well as block copolymer vesicles (or polymersomes) (9,10). Such delivery systems have demonstrated preferential accumulation in solid tumors compared to healthy tissue due to the enhanced permeation and retention effect (EPR) (3,11). The resulting therapeutic advantages include an enhanced antitumor effect and reduced systemic toxicities (3,12–14). Also, maximum tolerated doses of 5 to 10 fold greater than the free drug have been reported (12,15). In addition, the ability to overcome drug resistance has been reported (16,17). These and similar delivery systems, however, have had concerns. An early HPMA-DOX conjugate showed little, if any, improved efficacy in Phase I clinical trials compared to the free drug (18). Mucocutaneous toxicities were reported from liposomal delivery of DOX (19). And in a novel biodegradable delivery system not containing DOX, a polyglutamic acid carrier used with paclitaxol failed to demonstrate improved overall survival in Phase III clinical trials (20).

Gelatin is the denatured and partially hydrolyzed product of collagen (21). It has been used as a macromolecular carrier to deliver several drugs including amphotericin B (22), methotrexate (23), and tumor necrosis factor (24). It has also been shown to have cell uptake (25). Its high molecular weight and biodegradability are attractive properties for use as a carrier in a DOX macromolecular delivery system. A sufficiently high molecular weight can avoid glomerular filtration by the kidney leading to an extended circulation time and greater tumor accumulation by the EPR effect. Once the gelatin conjugate accumulates within the interstitial space of a tumor, its susceptibility to degradation by metalloproteinases, such as cathepsin B (25), would reduce the conjugate size and potentially enhance endocytotic uptake into the tumor cells. Recent reports describe encouraging results of high molecular weight HPMA-DOX conjugates containing cleavable links to allow breakdown in the body to lower molecular weight species (26,27). These lower sizes, however, are substantially larger than could occur with a biodegradable gelatin carrier. Thus, a more biodegradable gelatin conjugate of sufficient molecular weight may result in greater cell uptake of drug within the tumor while simultaneously prolonging circulation time.

Design of the macromolecular delivery system used in this investigation includes a pH dependent hydrazone bond covalently linking the drug and carrier. Little or no drug release is expected at neutral pH 7.4 within the circulation, but extensive release is expected at the acidic pH 4.8 of cellular lysosomes. After such an intracellular release, drug localization within cancer cells could produce enhanced efficacy and substantially reduced systemic drug toxicities. While pH dependent release of DOX using the hydrazone conjugate bond has been reported with several DOX delivery systems (4,8,28), the combination of this release mechanism with the biodegradability and high molecular weight of gelatin has not been reported. The overall goal of this investigation was to investigate the feasibility of such a delivery system. Its preparation and characterization, as well as its *in vitro* drug release, and cancer cell growth inhibition are reported.

MATERIALS AND METHODS

Materials

Type B bone gelatin was supplied by Kind and Knox (Gelita USA, Sargent Bluff, IA). Sephadex G50, Sephacryl HR 300, citraconic anhydride, 1-ethyl-3-(dimethylaminopropyl) carbodiimide HCl (EDC), glycylglycine benzyl ester p-toluene sulfonate (GGBE), hydrazine hydrate (Cat. No. 225819-50G), p-nitrobenzaldehyde, dimethylformamide,acetyl hydrazide, RPMI 1640, fetal bovine serum and phosphate buffered saline were obtained from Sigma-Aldrich (St. Louis, MO). Buffer, eluent salts, and syringe filters (0.2 μm pore, SFCA, Cat. No. 09-754-13) were obtained from Fisher (Pittsburgh, PA). Horse serum, EL4 mouse lymphoma cells, PC3 human prostate cells, and DMEM were from ATCC (Manassas, VA). Doxorubicin HCl was supplied by Bristol Myers Squibb (New York, NY)

Fractionation of Gelatin

About 300 mg of hydrated, heated, and dissolved gelatin in 10 mL of the eluent was fractionated through a 47 cm by 4.6 cm Sephracryl HR 300 column at 37°C at a flow rate of 1.7 mL/min using a 0.05 M ammonium acetate (pH 6.7) eluent and collected into 3.7 mL fractions. Fractions containing large amounts of the 100 kDa specie, identified by HPLC-SEC assay (see below), were combined and lyophilized. Several such first pass fractionations were combined and refractionated. Suitable second pass fractionations were then identified, combined, lyophilized, and refractionated for a third pass to obtain a mono disperse 100 kDa gelatin, designated fractionated gelatin

Preparation of Gelatin-Doxorubicin Conjugates

Gelatin granules (11.5% w/w moisture content) were hydrated, heated, and dissolved in 0.05 M sodium phosphate buffer at pH 8.5 to produce a stock gelatin solution from which 6.0 mL containing 100 mg of gelatin was used for preparation of G-Dox. Reactant quantities were based on one gram of gelatin containing 0.33 mmole amino groups, and 1.2 mmole carboxyl groups (21,29). Citraconic anhydride at a 16.8 molar ratio to gelatin amino groups (50 μL) was reacted with the gelatin solution at room temperature for 1 h. The reaction solution was then loaded onto a 41 cm by 0.5 cm SEC column packed with Sephadex G-50, eluted at 0.5 mL/min in 0.033 M MES buffer at pH 8.5 and 37°C, and the fractions containing the amino group blocked gelatin were collected, combined, lyophilized, and stored at −80°C. The lyophilized product was dissolved in 7.0 mL of water followed by addition of EDC at a 2.5 molar ratio to gelatin carboxyl groups (58 mg) and pH 7.0 was maintained. After 30 min, GGBE at a 2.5 molar ratio to gelatin carboxyl groups (119 mg) was added, and the reaction was maintained at pH 7.0 for an additional 2 h. The reaction solution was then adjusted to pH 8.5 and the gelatin product was collected by SEC and lyophilized as described above. Benzyl alcohol was hydrolyzed from the gelatin GGBE product after dissolution in water and pH adjustment to 8.5 for 2.5 hr followed by SEC collection and lyophilization as described above. Previous experiments established >94% hydrolytic release of benzyl alcohol from GGBE under these conditions. The gelatin product was again dissolved in water as above, followed by addition of EDC at a 6.3 molar ratio to the carboxyl groups (144 mg), and the reaction was maintained at

pH 7.0 for 30 min. Hydrazine hydrate at a 3.8 molar ratio to the carboxyl groups (27 μL) was added and the reaction solution was maintained at pH 7.0 for two additional hours. The gelatin product was collected by SEC and lyophilized as described above. The amino group blocking agent, citraconic acid, was removed from the gelatin product after dissolution of the gelatin in water as above and adjustment to pH 4.5 for 4 h, followed by SEC collection and lyophilization as described above except that the eluent was 0.005 M ammonium bicarbonate at pH 8.0. The gelatin product at this stage is designated Precursor. To add DOX, 10 mg of the Precursor, was dissolved in 5 mL of 0.05 M sodium bicarbonate, and adjusted to approximately pH 5.0. DOX (60 mg) dissolved in 1.43 mL of a 50:50 mixture of methanol:water was added slowly to Precursor and the reaction was maintained at pH 5.0 for 24 h in the dark. Afterwards, the reaction solution was adjusted to pH 8.0, and the conjugate (G-DOX) was collected by SEC in 0.005 M ammonium bicarbonate and lyophilized as described above. A second Precursor (10 mg) reaction with DOX was conducted in the same manner, and the resulting G-DOX was combined with the first batch by dissolution, SEC, and lyophilization prior to storage at −80°C. Conjugate prepared with fractionated gelatin (36 mg in 6 mL) utilized the same stoichiometry, but lower concentrations.

G-DOX Characterization

Determination of Hydrazide Addition

This procedure was modified from a previous report (30). Precursor was dissolved in 1.82 mL of 0.1 M acetate buffer (pH 5) at accurately prepared concentrations ranging from approximately 0.025 mg/mL to 0.20 mg/mL. To each solution, 0.180 mL of p-nitrobenzaldehyde (5 mM), dissolved in dimethylformamide, was added to obtain a concentration of 450 mM. These solutions, in microcentrifuge tubes, were placed in a water bath for 3 h at 37°C. Absorbance was measured at 340 nm and concentrations of hydrazone bonds formed with p-nitrobenzaldehyde were quantified using an extinction coefficient of 16,800 M^{-1} cm^{-1}. The extinction coefficient of the hydrazone chromophore was determined after synthesizing it from a reaction between p-nitrobenzaldehyde and acetylhydrazide, filtering the hydrazone precipitate, recrystallizing it in ethanol. This hydrazone was used to prepare a standard plot at concentrations ranging from 20–300 μM in 0.1 M acetate buffer (pH5) using absorbance measurements at 340 nm (31). Incomplete reaction of hydrazide groups (41%) with the reagent was determined from control experiments with acetylhydrazide measuring the extent of hydrazone formation with p-nitrobenzaldehyde after 3 h at 37°C at various molar ratios of reagent to hydrazide ranging from 5 to 100.

Determination of Drug Content

Accurately weighed G-DOX samples ranging from 2 to 5 mg were dissolved in 5.0 mL of 0.1 M potassium phosphate buffer at pH 4.8 for absorbance measurements at 488 nm using a Shimadzu UV-1800 spectrophotometer. Drug content was calculated as % w/w of the G-DOX sample using previously prepared linear calibration plots of DOX ranging from 0.0020 mg/mL to 0.040 mg/mL.

Determination of Molecular Weight and Size Distribution

HPLC-SEC assay of gelatin or G-DOX was a modification of a previously reported procedure (32). Samples were dissolved at a concentration of 0.5 mg/mL in sample solvent of 0.1 M sodium phosphate at pH 7.4 with 0.025% sodium azide, heated for 1 min at 65°C, and filtered with a 0.2 μm syringe filter for loading into inserts and vials. A Waters Millenium HPLC system with a BioSep S4000 SEC column (Phenomenex, Torrance, CA) and mobile phase of 0.1 M sodium phosphate at pH 7.4 with 0.5% sodium lauryl sulfate was run at 0.5 mL/min with a sample chamber temperature of 40°C and column temperature of 50°C for a 20 μL injection, a 30 min run time, and detection at 214 nm or 488 nm. Polystyrene sulfonate standards (Phenomenex, Torrance, CA) ranging from 10.6 kDa M_w to 282 kDa M_w were used to determine molecular weights. One standard was used with each gelatin or G-DOX assay to account for any shifts in peak retention times over the lifetime of the column. Calibration plots were constructed with the standards from several assays. A typical calibration plot prepared with five standards was $\log M_w = -0.153 \text{ min} + 4.19$ ($R^2 = 0.9916$). Higher M_w standards established the excluded specie size of about 310 kDa.

Release Studies

Design

Careful attention to containers, pH and DOX concentration should be used to minimize DOX complications of adsorption, degradation, and limited solubility (33). Stock solutions of G-DOX were prepared at 1.0 mg/mL in 0.03 M potassium phosphate with 0.12 M sodium chloride at pH 4.8, 6.5, or 7.4, or in cell growth medium, in 50-mL polypropylene centrifuge tubes. Three or four replicates of 110 μL G-DOX solution were placed in independent siliconized polypropylene microcentrifuge tubes at 37°C, and 100 μL samples were collected at 0, 3, 9, 24, 48 h into new microcentrifuge tubes for sample preparation and assay.

Assay of Released DOX

A modified doxorubicin extraction procedure was used (34). Drug release samples (100 μL) were spiked with 100 μL of 0.025 mg/mL daunorubicin as the internal standard. Cold ethanol (1 mL) was added followed by 10 min of centrifugation at 12000×G. The supernatant was transferred to a glass centrifuge tube, and 200 μL of 1 M phosphate buffer at pH 8.5 followed by 2.8 mL dichloromethane was added. The glass tube was closed by a screw cap with PTFE liner, and shook for 10 min followed by 5 min centrifugation at 1600×G. The upper aqueous layer was removed by vacuum suction. The lower organic layer was transferred to a glass culture tube sitting in a 30°C water bath, and evaporated to dryness by nitrogen gas. Calibration plots of DOX standards ranging from 0.0010 to 0.10 mg/mL were prepared using the same extraction procedure but included 1.0 mg/mL gelatin. Samples and standards dried by evaporation were stored at −80°C before analysis. For HPLC analysis, samples and standards were reconstituted in 100 μL of 0.03 M potassium phosphate with 0.12 M sodium chloride at pH 4.8, and centrifuged at 1600 × G for 2 min. The supernatant (80 μL) was transferred to a glass HPLC vial insert, and 45 μL was analyzed using a Shimadzu HPLC system composed of a Zorbax C18 column (Agilent, Santa Clara, CA) a mobile phase of 10 mM sodium phosphate buffer with 10 mM triethylamine (pH 3.5) at 75:25 with acetonitrile, a 1 mL/min flow rate, detection at 235 nm, and run time of approximately 10 min. Samples were bracketed and determined by two 5-point daily calibration plots. The correlation coefficients of calibration plots were greater than 0.99.

Cell Growth Inhibition Studies

Cell Cultures

EL4 Mouse lymphoma cells were cultured in DMEM with 10% horse serum. PC3 Human prostate cells were cultured in RPMI 1640 with 10% fetal bovine serum. Cells were maintained at 37°C under 5% CO_2 and 95% relative humidity without antibiotics. During growth inhibition studies antibiotics were added at 100 units/mL penicillin and 100 μg/mL streptomycin. The presence of antibiotics did not affect determination of growth inhibition by DOX (not shown). Cells were used between passages 3 to 11.

EL4 Mouse Lymphoma Cell Growth Inhibition

Cells were seeded for replicates of five in a 96-well plate at 1×10^4 cells/mL with 200 μL of cell suspension. A second plate of cells was seeded as a 24 h control of cell growth prior to drug addition. Both plates were incubated at 37°C under 5% CO_2 and 95% relative humidity. After 24 h, 50 μL of growth medium containing agent was added to wells to produce incubating equivalent DOX concentrations ranging from 0.001 to 5 μM DOX, 0.01 to 18 μM G-DOX, or 0.05 to 100 μM Precursor (equivalent gelatin concentration) for 48 h. Growth medium without agent was added to untreated cells. Cell growth was measured with 25 μL of AlamarBlue (Invitrogen, Carlsbad, CA) per well, a 6 h incubation, and fluorescence measurements with excitation at 530 nm and emission at 590 nm. Two experiments were conducted for DOX and G-DOX, one experiment for the Precursor. Growth inhibition was calculated by subtracting the 24-hour cell growth value from the treated and untreated cells and expressing the result as a percent growth of untreated cells. Growth inhibition lines were calculated by nonlinear regression with Sigma Plot Regression Wizard using a four-parameter logistic curve: $y = \min + (\max - \min)/[1 + (x/IC_{50})^{-Hillslope}$ where x is concentration and y is percent growth, and the IC_{50}, Hillslope, min and max parameters were obtained from regression analysis of the overall curve. The Precursor line was calculated with a quadratic equation.

PC3 Human Prostate Cell Growth Inhibition

Cells, and 24 h controls, were seeded as above except at 2×10^4 cells/mL. DOX or G-DOX was added as above to produce incubating equivalent DOX concentrations ranging from 0.01 μM to 100 μM and 0.001 to 20 μM, respectively for 72 h. Cells were then washed with PBS and incubated with 250 μL of 10% AlamarBlue in growth medium for 3 h followed by fluorescence measurement of excitation at 560 nm and emission at 590 nm. Three separate experiments were conducted for each agent. Growth inhibition graphs and IC_{50} values were calculated as above.

Statistics

Tests of statistically significant differences were conducted with a *t*-test for equal sample size and unequal variance at $p \leq 0.05$.

RESULTS

Preparation and Characterization of G-DOX

The preparation of G-DOX, structure shown in Fig. 1, involves several steps of pH controlled reaction, SEC separation, and lyophilization. The amino group blocking agent citraconic anhydride is used to prevent crosslinking during the carbodiimide (EDC) reactions between gelatin carboxyl and amino groups while allowing such reactions between the growing gelatin product and the respective reagent. The

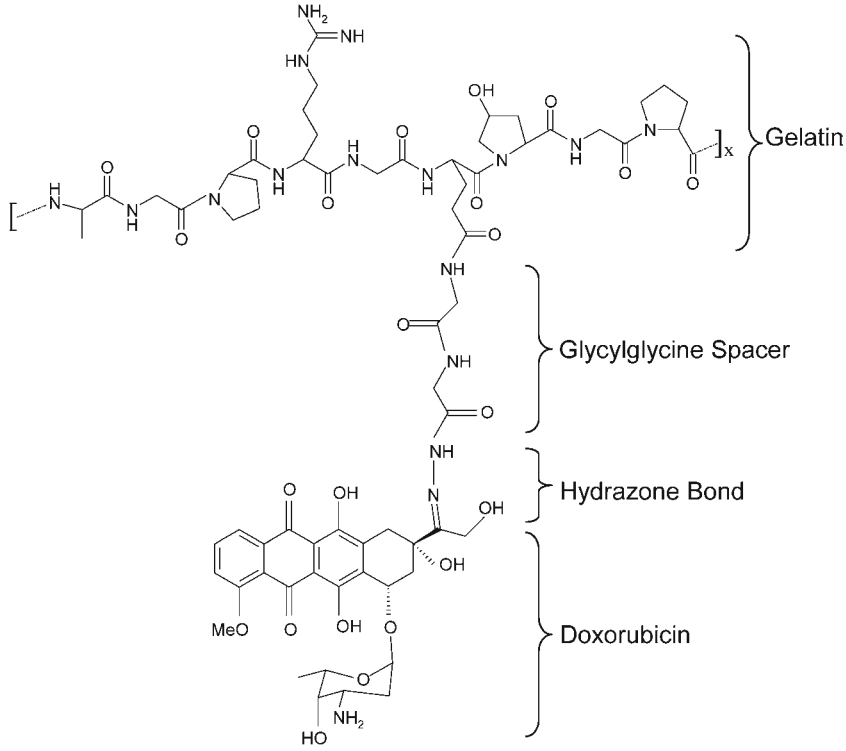

Fig. 1 Structure of gelatin – doxorubicin conjugate (G-DOX). Components of the conjugate are identified as carrier, spacer, acid-labile conjugate bond and drug.

spacer, GGBE, has a benzyl ester group as a carboxyl blocking group to allow its amino group to react with the EDC activated gelatin carboxyl groups. The blocked carboxyl group prevents multiple spacer self-reactions and maintains a spacer length of a single dipeptide. Hydrolytic release of this carboxyl blocking group (benzyl alcohol), and of the amino blocking group take place in separate steps. The hydrazine reaction introduces the source of the conjugate bond, after its subsequent reaction with DOX forming a pH dependent hydrazone bond in the final step.

Measurements of reaction extent during G-DOX preparation are shown in Table I for the batches used in the respective studies. It should not be overlooked that these assays also confirm the presence of the respective groups.

Table I Chemical Characterization of Gelatin: Doxorubicin Conjugates

Conjugate Batch	Studies used in	Hydrazide (mmole/g)	DOX[a]	Hydrazide (% of gelatin COOH groups)[b]	DOX
G-DOX1	Release	0.56 ± 0.02	5.0	47 ± 2	7.2
G-DOX2[c]	EL4 Cells	0.37 ± 0.02*	3.4	31 ± 1*	4.9
G-DOX3	PC3 Cells	0.56 ± 0.02	3.7	47 ± 2	5.3
Precursor[d]	EL4 Cells	0.56 ± 0.02	–	47 ± 2	–

mean ± SD, $n = 3-4$

[a] % w/w, $n = 1$

[b] calculated from 1.2 mmoles COOH groups per g of gelatin (Ref 21)

[c] prepared with fractionated gelatin, also used for release

[d] before addition of DOX to form G-DOX1. *$p < 0.05$

The amounts are expressed as mmole/g to account for the variation of molecular weight during preparation (see below). The amounts are also expressed as a percent of the gelatin carboxyl groups that are sites of the addition steps to prepare G-DOX. The hydrazide content on Precursor and G-DOX, except for G-DOX prepared with fractionated gelatin, corresponds to 47% of the gelatin carboxyl groups. The lower hydrazide content on G-DOX prepared with fractionated gelatin is due to the lower starting amount of gelatin, and the resulting lower reagent concentrations. DOX content is 5–7% of gelatin carboxyl groups, or 3.4 – 5.0% w/w.

The size reductions during preparation of G-DOX3 are shown by the chromatograms in Fig. 2. The starting gelatin molecular weight distribution (Fig. 2a) is polydisperse, with species ranging from 10 kDa (~19 min) to≥310 kDa (~11 min). The α-chain specie of 100 kDa (14 min) is most abundant, and the β-chain specie of about 180 kDa (12 min), and possibly the γ-chain specie (ca 300 kDa) are also detectable. The high molecular weight species≥ 310 kDa are combined in the exclusion species peak at about 11 min. The 24 min peak represents the azide and phosphate species. The first step in preparation, the blocking of gelatin amino groups with citraconic anhydride, does not alter the molecular weight distribution of starting gelatin. The chromatogram of this blocked gelatin (not shown) is identical to Fig. 2a. Figure 2b represents the amino group blocked gelatin bound to GGBE. The GGBE addition step using EDC shows a substantial shift towards species of lower molecular weight, notably the 10 kDa

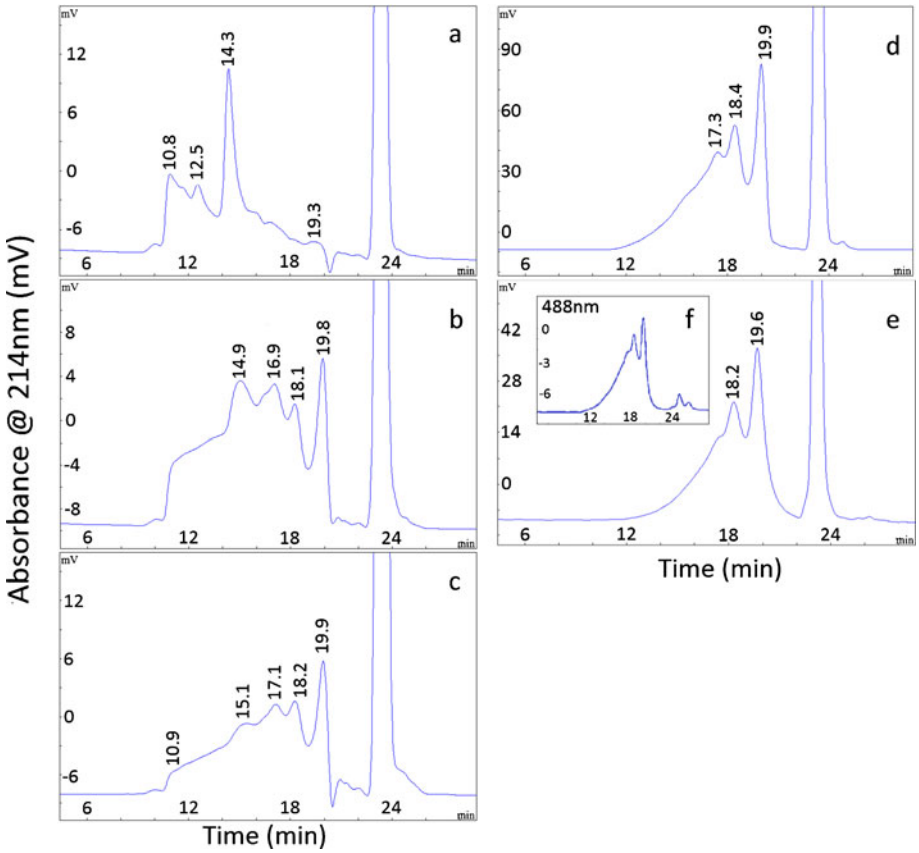

Fig. 2 SEC chromatograms showing changes in size and molecular weight distribution during preparation of G-DOX. (**a**) starting gelatin, (**b**) amino group blocked gelatin with GGBE, (**c**) amino group blocked gelatin with glycylglycine, (**d**) gelatin glycylglycine with hydrazine (Precursor), (**e**) G-DOX, (**f**) G-Dox measured at 488 nm. See text for specifics of synthesis and SEC.

specie. Figure 2c shows the result of benzyl alcohol hydrolysis from the GGBE spacer to generate carboxyl groups, accompanied by an additional shift towards lower molecular weight. Hydrazine addition using EDC (not shown) is followed by citraconic group removal to produce Precursor shown in Fig. 2d. This chromatogram is virtually identical to the one not shown after hydrazine addition. It can be seen that the EDC reaction for hydrazine addition also produces a substantial shift to lower molecular weights. The completed conjugate shown in Fig. 2e shows a small shift from Precursor after the 24 hr DOX addition step. The inset Fig. 2f shows G-Dox measured at 488 nm and confirms incorporation of DOX. G-DOX has a binodal distribution of about 30 kDa and 10 kDa, for a weighted average molecular weight of 22 kDa. No difference in molecular weight and distribution was found between G-DOX prepared with unfractionated or fractionated gelatin (data not shown).

Drug Release

The *in vitro* release of DOX at various pH in 0.03 M phosphate buffer with 0.12 M NaCl at 37°C for up to 48 h is shown in Fig. 3. A small burst effect of 4 to 6% of the drug load is measured at zero time. At pH 4.8, a rapid release occurs within 8 h with a maximum release of 48% measured by 24 h. At pH 6.5 a gradual increase produces a 22% release by 48 h, while at pH 7.4 a 10% maximum release is

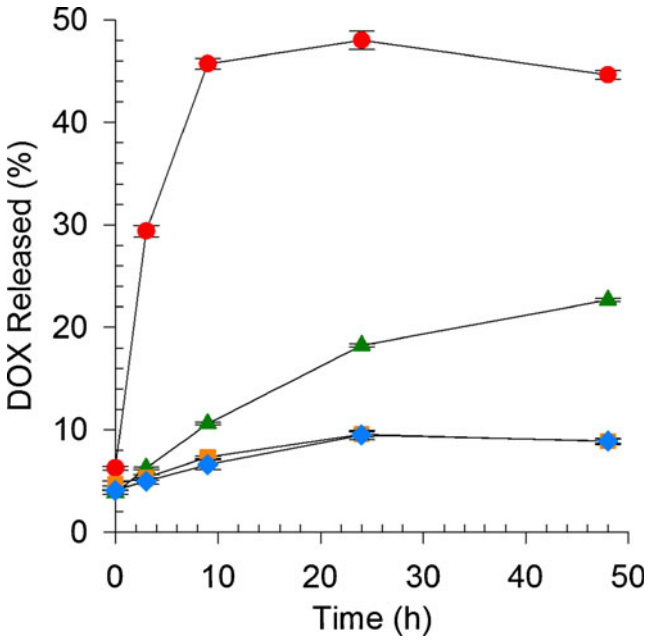

Fig. 3 pH dependent release of DOX from G-DOX. Key (●) pH 4.8, (▲) pH 6.5, (■) pH 7.4 in 0.03 M potassium phosphate with 0.12 M sodium chloride. (♦) growth medium of DMEM with 10% horse serum at pH 7.4. *Symbols* represent mean ± SD.

observed by 24 h. Release of DOX is also shown in cell growth medium, but is indistinguishable from release in buffer at pH 7.4. Differences of release between these two are within 1%. The differences between DOX release from G-DOX prepared with unfractionated or fractionated gelatin are within 2% (data not shown).

Cell Growth Inhibition

Conjugate prepared from unfractionated or fractionated gelatin was considered equivalent for growth inhibition studies because molecular weight and distribution, as well as DOX release, was the same for both conjugates. Conjugate induced growth inhibition of cancer cells is shown in Fig. 4a and b. Growth inhibition of EL4 mouse lymphoma cells is in the order of Dox > G-DOX > Precursor. The free drug is also more effective than G-DOX against PC3 human prostate cells. The G-DOX IC_{50} value in EL4 cells of 0.26 μM is 9 times greater than the IC_{50} value of Dox (see Table II). The G-DOX IC_{50} value in PC3 cells of 0.77 μM is 3.2 times greater than that of DOX. The negative cell growth values for the highest DOX concentrations indicate a drug induced reduction of cell concentrations.

DISCUSSION

This study is an investigation of the feasibility of a biodegradable and pH dependent delivery system for DOX with potential therapeutic advantages of enhanced tumor accumulation and substantially reduced myocardial as well as reduced systemic toxicities. Measurement of the extent of addition reactions allows assessment of the conjugate preparation. Hydrazide sites available for drug addition represent 47% of the gelatin carboxyl groups. A DOX content of 5.0% w/w, or 7% of the gelatin carboxyl sites, represents about a 15% DOX addition yield on the hydrazide sites. The DOX load is comparable to levels reported in similar conjugates ranging from 3.2% w/w in a pullulan – DOX conjugate (8) to 8.6% w/w in a HPMA – DOX conjugate (27).

The size reduction during preparation occurs substantially from the two carbodiimide (EDC) reaction steps (see Fig. 2b and d). Experiments on gelatin with similar reaction conditions of pH, temperature, buffers, and repetitions of SEC and lyophilization, produced substantially less size reductions in the absence of EDC (data not shown). It is unclear of the role, if any, that this common protein reagent has in gelatin degradation during these reactions.

Because SEC measures only size, an alternative explanation for these size reductions was considered. Intramolecular crosslinking might occur during EDC reactions with the few

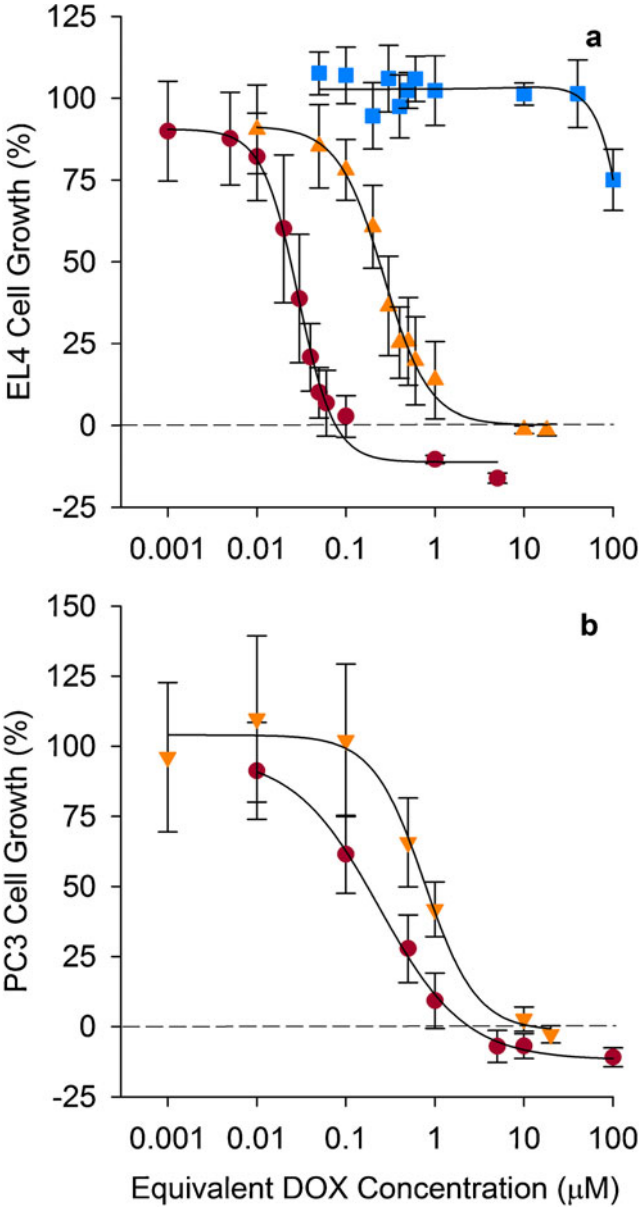

Fig. 4 Growth inhibition profiles of G-DOX in two cancer cell lines. (**a**) EL4 mouse lymphoma cells; (**b**) PC3 human prostate cells. Key: (●) DOX, (▲) G-DOX from fractionated gelatin, (▼) G-DOX from unfractionated gelatin, (■) Precursor at equivalent gelatin concentration. *Symbols* represent mean ± SD.

Table II IC_{50} Values for Doxorubicin and G-DOX in EL4 Mouse Lymphoma and PC3 Human Prostate Cancer Cells

Cell Line	DOX (μM)	G-DOX[a] (μM)
EL4	0.029 ± 0.005	0.26 ± 0.07*
PC3	0.24 ± 0.13	0.77 ± 0.46*

Mean ± SD of 2 to 3 experiments with replicates of 5 wells. Statistically significant difference from respective DOX value at $*p < 0.05$

[a] fractionated gelatin used to prepare G-DOX for EL4 cells

(~5%) initially unblocked, and possibly more amino groups if such blocking gradually hydrolyses during the two EDC reactions at pH 7. This crosslinking could conceivably constrict conformation of the gelatin molecule to a smaller size. Additional experiments were conducted in the following way to explore this possibility. Gelatin (with unblocked amino groups) at a concentration below that which would have any significant intermolecular entanglements was reacted with 4 fold higher EDC amounts than used in G-Dox preparation (*i.e.* a 10-fold molar excess of gelatin carboxyl groups). In this manner, only intramolecular reactions would occur. If such crosslinking was induced, the size reductions would be detectable by SEC measurements. These measurements showed little difference from that of starting gelatin. Based on these results, and the presence of amino group blocking, it was concluded that chemical degradation, and not intramolecular crosslinking, produced the observed shifts to smaller sizes.

It is difficult to predict if the G-DOX size of 22 kDa is large enough to avoid glomerular filtration. The cut-off for this has been reported at about 40 kDa (35) but this value is complicated by random coil *versus* native protein conformation, charge, and deformability to name a few of these factors. This size, however, is similar to the sizes of earlier HPMA – DOX conjugates of 24 kDa (36) and 26 kDa (4). These synthetic polymer and gelatin conjugates are substantially larger than free DOX and are anticipated to have therapeutic advantages of passive accumulation within solid tumors due to the EPR effect. However, in contrast to the synthetic polymer conjugates, the gelatin component of G-DOX would be susceptible to cathepsin B degradation (25) to lower molecular weight fragments within solid tumors with the potential to enhance endocytotic cell uptake.

The pH dependent DOX release profiles of G-DOX in Fig. 3 are similar to release profiles in other hydrazone macromolecular DOX conjugates (4,8,37). The limited DOX release at pH 7.4 and in growth medium is intended to produce few drug interactions with healthy tissue within the systemic circulation, in particular the myocardial cells. Release at the acidic lysosomal pH of 4.8 is intended to produce a localized drug concentration within cancer cells, after EPR induced tumor accumulation and endocytotic uptake of conjugate fragments into the cancer cell. The relatively small drug release at the interstitial pH of 6.5 indicates that some drug could be released within the tumor but outside the cancer cell.

The EL4 mouse lymphoma cells have a suspension morphology in cell culture but can induce lymphoma tumors after injection in mice. PC3 human prostate cancer cells have an adherent morphology and form a solid tumor that currently has no effective chemotherapy. The large number of unreacted hydrazide groups in G-DOX raises the possibility of these groups inducing unwanted toxicity on healthy tissue. Less than 1000-fold, if any, of such toxicity was observed from the Precursor on EL4 cells (see Fig. 4). It is interesting to note that the negative cell growth values at high DOX concentrations indicating a bactericidal action are not shown at equivalent G-DOX concentrations. The limited release of only 10% DOX in growth medium provides a partial, but incomplete explanation of this reduced effect.

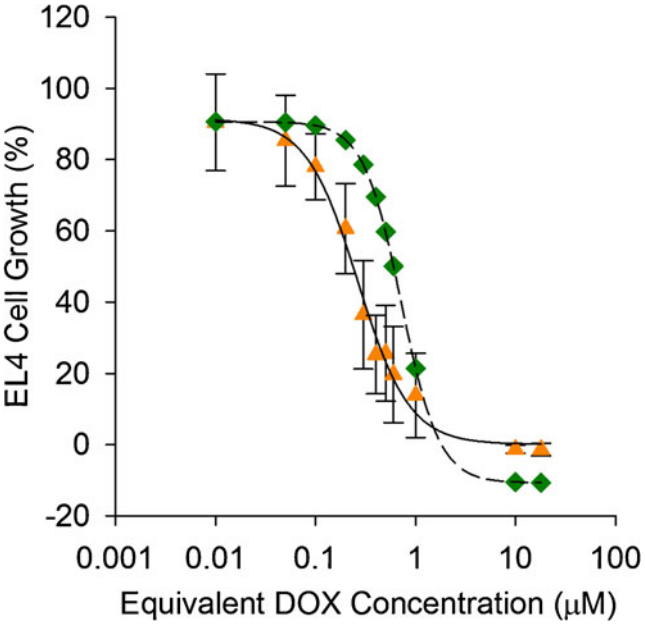

Fig. 5 Comparison of the calculated DOX burst release effect (♦) and G-DOX (▲) on growth inhibition of EL4 cells.

The burst release from adsorbed DOX very likely contributes to the measurements of growth inhibition by G-DOX. The contribution of free DOX can be estimated as follows. From the 4% burst release at time zero in growth medium (Fig. 3), the growth inhibition at corresponding concentrations of burst release DOX from G-DOX can be calculated from the growth inhibition equation parameters. This calculated growth inhibition is shown in Fig. 5 compared to the observed EL4 growth inhibition of G-DOX (from Fig. 4). The results indicate that G-DOX growth inhibition exceeds that of the free DOX. The mechanism for this G-DOX growth inhibition in growth medium at pH 7.4 is unclear but may include an effect from the small amount of released DOX in excess of burst release (about 5%), as well as cellular uptake of G-DOX and release of DOX from the acidic lysosomes.

CONCLUSIONS

A novel biodegradable gelatin-doxorubicin conjugate of about 22 kDa designed for passive accumulation in solid tumors by the EPR effect has been prepared. A near 50%

drug release at the lysosomal pH of 4.8, but only 10% release at pH 7.4 was achieved with this conjugate which demonstrates the potential therapeutic advantages of tumor cell accumulation and little effect on healthy tissue. The conjugate demonstrated growth inhibition on an EL4 lymphoma cell line of a suspension morphology and on a PC3 prostate cancer cell line of an adherent morphology. Studies are in progress on a higher molecular weight G-DOX.

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